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# Defining Dynamic Protein Interactions Using SILAC-Based Quantitative Mass Spectrometry

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## Abstract

Protein–protein interactions are essential to various physiological processes in living cells. A full characterization of protein interactions is critical to our understanding of their roles in the regulation of protein functions. Affinity purification coupled with mass spectrometry (AP-MS) has become one of the most effective approaches to systematically study protein–protein interactions. In combination with quantitative mass spectrometry, specific interacting proteins can be efficiently distinguished from nonspecific background proteins. Based on interaction affinity and kinetics, protein interactions can be classified into different categories such as stable and dynamic interactions. Standard biochemical methods are effective in capturing and identifying stable protein interactions but are not sufficient enough to identify dynamic interactors. In this chapter, we describe integrated strategies to allow the identification of dynamic interactors of protein complexes by incorporating new sample preparation methods with SILAC-based quantitation.

### Keywords

AP-MS; Protein; protein interaction; Quantitative mass spectrometry; HB-tag; Dynamic interactors; Stable interactors; Proteasome interacting proteins; PAM-SILAC; MAP-SILAC; Tc-PAM SILAC

## 1 Introduction

Protein complexes are dynamic and functional entities that are of critical importance for various biological processes in living cells. Protein–protein interactions play key regulatory roles in controlling the assembly, structure, and function of protein complexes in response to diverse cellular cues [1]. It has been well recognized that aberrant protein–protein interactions can lead to various human diseases including cancer [2, 3]. Therefore, a comprehensive characterization of interaction networks of protein complexes not only improves our understanding of cellular processes but also provides potential targets for future therapeutics. Due to technological advancement in recent years, affinity purification–mass spectrometry (AP-MS) has become the method of choice for globally mapping protein–protein interactions from various organisms with speed and sensitivity [4–6]. In combination with quantitative mass spectrometry, highly reliable interaction data can be

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obtained in which specific protein interactors can be effectively distinguished from nonspecific background proteins [6,7]. This is important since nonspecific binding to the affinity matrix cannot be completely eliminated in resin-based affinity purification processes.

Although various quantitative mass spectrometry methods can be incorporated with AP-MS strategies, the stable isotope labeling of amino acids in cell culture (SILAC) strategy appears to be more attractive owing to global protein labeling during cell culture prior to any sample preparation procedures, thus minimizing sample loss during AP-MS experiments [7,8]. When the standard SILAC approach is used, cells expressing the tagged bait protein are labeled metabolically with the light isotope and control cells (e.g., cells expressing the tag alone) are metabolically labeled with the heavy isotope or vice versa. For simplicity, in the following text, only the former situation will be described. After metabolic labeling and cell lysis, equal amounts of light and heavy labeled cell lysates are mixed before purification. We term this kind of standard SILAC strategy PAM (*p*urification *a*fter *m*ixing)-SILAC as shown in Fig. 1a [9]. After purification, the samples are subject to digestion and mass spectrometry analysis. When the purified proteins are present in both light and heavy labeled forms, their resulting peptides will be detected in MS as peptide pairs (light *vs*. heavy) with defined mass differences depending on the number of stable isotope-labeled amino acids in each peptide. By comparing mass spectral peak intensities of peptide pairs, their relative abundance ratios (i.e., SILAC ratios = light/heavy) can be calculated, which are the basis for distinguishing specific proteins from nonspecific background. Since the abundance of a specific interacting partner purified from the tagged bait sample should be significantly higher than the one from the control, its SILAC ratio would be much higher than 1. The higher the ratios, the more specific the interactions are. In contrast, the abundance of nonspecifically bound background proteins should be comparable from both the sample and the control, resulting in their SILAC ratios close to 1. Thus, specifically interacting proteins can be determined quantitatively using the PAM-SILAC method (the original SILAC approach).

In addition to specificity, proteins interact with each other with different affinity and kinetics. Only protein interactions with high enough affinity can be preserved during AP-MS experiments due to extensive washing steps. Among these interactions, proteins that interact with the bait at fast on and slow off rates are considered as stable interactors, whereas proteins that interact with the bait at fast on/off rates are known as dynamic interactors. With the PAM-SILAC method, protein purification is carried out after mixing the cell lysates from two types of cells (sample *vs*. control) that have been differentially labeled; all proteins are present in both the light (sample) and heavy (control) labeled forms during the purification. Although the presence of the two differentially labeled cell lysates does not affect stable interactions, it does interfere with the interactions between the dynamic interactors and the bait. As a result, some of the light labeled dynamic interactors initially bound to the bait can be replaced by their corresponding heavy labeled forms from the control cell lysate, thus leading to decreased SILAC ratios and hampering their identification as specific interactors. Depending on the interaction kinetics, an equilibrium can be achieved between the two differentially labeled forms of the dynamic interactors that are bound to the

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bait at a given incubation time, which will decrease SILAC ratios of these interactors close to those of background proteins. Thus, these specific but dynamic interactors cannot be effectively distinguished from background proteins based on their SILAC ratios determined by the original SILAC approach. Therefore, the PAM-SILAC method is not best suited for unambiguous identification of dynamic interactions. To circumvent this problem, we have developed the Tc (Time controlled)-PAM- SILAC method [9], in which different incubation times (e.g., 20 min, 1 h, 2 h) can be selected to facilitate the identification of dynamic interactors (Fig. 2). This is based on the observations that SILAC ratios for dynamic interactors are dependent on incubation time and they increase with less incubation time due to decreased interaction exchange between the light and heavy labeled proteins. If the on/off rates are not too fast, the dynamic interacting partners can be identified with shortened incubation times. Since stable interactors have SILAC ratios independent of incubation time, stable and dynamic interactors can be distinguished by the Tc-PAM-SILAC method. Although effective, the Tc-PAM- SILAC method may not be sufficient to identify dynamic interactors with very fast on/off rates. This is due to the fact that: (1) there is a limit on experimentally feasible incubation time; and (2) shortened incubation time often sacrifices binding efficiency and thus leads to compromised sensitivity.

In order to quantitatively identify all of the dynamic interacting proteins with different on/off rates, we have further developed a new sample preparation strategy, MAP (*m*ixing *a*fter *p*urification)-SILAC, which allows the complete elimination of interaction interferences from proteins in control cell lysates during purification (Fig. 1b). In the MAP-SILAC strategy, protein purification is carried out separately from equal amounts of the two cell lysates to be compared (sample *vs*. control) that have been differentially labeled. After the purification, the purified protein complexes are mixed for digestion and MS analysis. With this approach, there is no interaction exchange between differentially labeled forms and dynamic interactors can preserve their high SILAC ratios for unambiguous identification as specific interacting proteins. By comparing protein SILAC ratios obtained from MAP-SILAC and PAM-SILAC experiments, dynamic and stable interactors can be effectively distinguished [9, 10]. These new methods significantly expand the ability of AP-MS strategies to study protein interactions, allowing not only the identification of important but previously unidentifiable interacting proteins, but also the characterization of the nature of protein interactions.

These new integrated strategies have been successfully applied to characterize proteasome- [9] and COP9 signalosome- interacting proteins [10]. In this chapter, we use the study of dynamic interacting proteins of the human 26S proteasome complex as an example to illustrate the experimental workflow. The 26S proteasome is a multi-catalytic proteinase complex responsible for ubiquitin/ATP-dependent protein degradation [11]. His-Bio- (HB-) tag based affinity purification strategy is employed to isolate the human proteasome complex in a single step [12, 13]. In combination with MAP-SILAC and Tc-PAM-SILAC, a number of dynamic interactors of the 26S proteasome have been identified, most of which are key regulators in the ubiquitin–proteasome degradation system [9]. This further demonstrates the critical importance of identifying biologically significant dynamic interactors of protein complexes.

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## 2 Materials

### 2.1 Cell Culture and Metabolic Labeling

1. A HEK293 cell line stably expressing C-terminal HTBH-tagged Rpn11: $293^{Rpn11\text{-}HTBH}$ (*see* **Note 1**).
2. A HEK293 cell line stably expressing the HTBH-tag alone: $293^{HTBH}$.
3. Culture medium: EMEM (deficient in lysine and arginine) (e.g., from Sigma-Aldrich) (*see* **Note 2**).
4. Heavy isotope-labeled amino acids: $^{13}C_6{}^{15}N_4$-Arginine and $^{13}C_6{}^{15}N_2$-lysine. Make two 100× stock solutions: 2.8 mg/ml arginine and 7.3 mg/ml lysine in sterile water.
5. Light isotope-labeled amino acids: $^{12}C_6{}^{14}N_4$-arginine and $^{12}C_6{}^{14}N_2$-lysine. Make two 1,000× stock solutions: 28 mg/ml arginine and 73 mg/ml lysine in sterile water.
6. Heavy SILAC medium: EMEM supplemented with 28 μg/ml $^{13}C_6{}^{15}N_4$-arginine, 73 μg/ml $^{13}C_6{}^{15}N_2$-lysine, 10 % dialyzed fetal bovine serum, and 1 % penicillin/streptomycin (the 100 % solution contains 10,000 U penicillin and 10,000 U streptomycin).
7. Light SILAC medium: EMEM supplemented with 28 μg/ml $^{12}C_6{}^{14}N_4$-arginine, 73 μg/ml $^{12}C_6{}^{14}N_2$-lysine, 10 % dialyzed fetal bovine serum, and 1 % penicillin/streptomycin.

### 2.2 HB-tag Based Affinity Purification for MAP-SILAC and Tc-PAM- SILAC Experiments

1. 1× protease inhibitor cocktail: 1 μg/ml phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin. Make a 100× stock solution for phenylmethylsulfonyl fluoride, store at 4 °C, and 1,000× stock solutions for leupeptin, aprotinin, and pepstatin, store at -20 °C.
2. 1× phosphatase inhibitor cocktail: 5 mM NaF, 0.1 mM $Na_3 VO_4$, 2.5 mM $Na_4P_2O_7$, 1 mM EDTA. Make a 10× stock solution; store at –20 °C.
3. Trypsin–EDTA.
4. PBS.
5. Lysis buffer A:100 mM sodium chloride, 50 mM sodium phosphate, 10 % glycerol, 5 mM ATP, 1 mM DTT, 5 mM $MgCl_2$, 1× protease inhibiter cocktail, 1× phosphatase inhibitor cocktail, 0.5 % NP-40, pH 7.5. Make the buffer right before the experiment.
6. 20 gauge needles.
7. ImmunoPure Streptavidin (Thermo Scientific).
8. TEB buffer: 50 mM Tris–HCl, pH 7.5.
9. AcTEV protease (Life Technologies).
10. Microspin column (Bio-Rad).

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11. Siliconized tubes (Axygen).

### 2.3 Protein Digestion and LC-MS/MS Analysis

1. Trichloroacetic acid (TCA), 100 % (w/v): dissolve 100 g TCA into 70 ml $H_2O$ and keep at 4 °C.
2. Acetone, keep at −20 °C.
3. 50 mM $NH_4HCO_3$ with 8 M urea.
4. Trifluoroacetic acid (TFA).
5. 1 μg/μl sequencing-grade endopeptidase LysC stock solution.
6. 0.4 μg/μl sequencing-grade trypsin stock solution: dissolve 20 μg of trypsin in 50 μl of 1 mM TFA (*see* **Note 3**).
7. 10 % formic acid.
8. 2.1 mm × 10 cm PolySULFOETHYL A column (Nest Group).
9. Strong cation exchange (SCX) chromatography: AKTA Basic 10 (GE Healthcare).
10. AKTA buffer A: 5 mM $KH_2PO_4$, 0.1 % formic acid, 30 % acetonitrile, pH 2.7. For 1 l of the solution, add 0.68 g $KH_2$ $PO_4$ and 1 ml of formic acid to 700 ml water, adjust the pH with formic acid, filter the solution using a 0.45 μm filter, and then add 300 ml of acetonitrile. The solution needs to be degassed for 20 min.
11. AKTA buffer B: 5 mM $KH_2PO_4$, 0.1 % formic acid, 30 % acetonitrile, 300 mM KCl, pH 2.7. For 1 l of the solution, add 0.68 g $KH_2PO_4$, 1 ml of formic acid, and 26.1 g KCl to 700 ml $H_2O$, adjust the pH with formic acid, filter the solution using a 0.45 μm filter, then add 300 ml of acetonitrile. The solution needs to be degassed for 20 min.
12. Vivapure C18 microspin columns (Vivascience).
13. NanoLC capillary column (75 μm ID × 150 mm long) packed with Polaris C18-A resin (Varian Inc.).
14. Mass spectrometer: QSTAR XL MS (AB Sciex) (*see* **Note 4**).
15. Nano LC solvent A: 2 % acetonitrile, 0.1 % formic acid in $H_2O$.
16. Nano LC solvent B: 98 % acetonitrile, 0.1 % formic acid in $H_2O$.

### 2.4 Database Searching for Protein Identification and Quantification

1. LC-MS/MS data extraction: instrument specific scripts from the manufacturer.
2. Protein identification and quantitation software: Protein Prospector (University of California, San Francisco).

### 2.5 Validation of Dynamic Interactions Using Quantitative Western Blotting

1. Horseradish peroxidase (HRP)-conjugated secondary antibody.

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2. Super Signal West Pico chemiluminescent substrate (Pierce/Thermo Fisher Scientific).
3. Anti-Rpt6 antibody (BioMol).
4. Anti-FLAG antibody (Sigma-Aldrich).
5. Odyssey infrared scanning system (LI-COR Biosciences).
6. pcDNA/FRT-ADRM1-FLAG.
7. TuboFect transfection reagent (Thermo scientific).
8. Protein assay kit (Bio-Rad).
9. 10 % SDS-PAGE gel.
10. PVDF membrane (Bio-Rad).
11. Wet/tank blotting system (Bio-Rad).
12. Stripping buffer: 5 mM $KH_2PO_4$, 0.1 % formic acid. For 1 l of the solution, add 0.68 g $KH_2PO_4$ and 1 ml of formic acid to 999 ml water.
13. Cy5-conjugated anti-mouse IgG (Invitrogen).

## 3 Methods

### 3.1 Cell Culture and Metabolic Labeling

1. Culture $293^{Rpn11\text{-}HTBH}$ cells in light SILAC medium. When cell culture changes from regular medium to SILAC medium, cells need to be grown for more than seven cell doublings to ensure complete labeling. Then grow cells to about 90 % confluence prior to cell lysis.
2. Culture $293^{HTBH}$ cells (control cell line) in heavy SILAC medium. Cells need to be grown for more than seven cell doublings in heavy SILAC medium to ensure complete labeling. Then grow cells to about 90 % confluence prior to cell lysis.
3. For label-switch experiments, culture $293^{Rpn11\text{-}HTBH}$ cells in heavy SILAC medium and $293^{HTBH}$ cells in light SILAC medium.

### 3.2 HB-tag Based Affinity Purification

1. Trypsinize cells and wash them three times with 1× PBS buffer.
2. Collect cell pellets and lyse cells using lysis buffer A by pushing the lysate ten times through a 20 gauge needle.
3. Centrifuge the lysates at maximum speed of a microcentrifuge for 15 min to remove cell debris, and incubate the supernatant with 25 μl of Streptavidin resin per plate for the desired amount of time at 4 °C (*see* **Note 5**).
4. Wash the Streptavidin beads with 20 bed volumes of lysis buffer A without protease and phosphatase inhibitors (*see* **Note 6**).
5. Wash the beads with 10 bed volumes of TEB buffer.

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6. Incubate the beads in 2 bed volumes of TEB buffer with 1 % TEV at 30 °C for 1 h with rotation (*see* **Note 7**).

7. Elute the human 26S proteasome complex from the beads by passing the mixture through a Bio-Rad microspin column (*see* **Note 8**).

### 3.3 Protein Digestion and LC-MS/MS Analysis

To avoid keratin contamination in your samples, you need to wear a hair net, sleeves, and clean gloves for the following procedure.

1. Precipitate purified complexes by adding TCA to a final concentration of 25 %, place the mixture on ice for 1 h. Spin at maximum speed for 15 min. Remove the supernatant. Wash the pellet in 1 ml of ice-cold acetone and centrifuge for 15 min, repeat the washing step two more times (*see* **Note 9**).

2. Redissolve the pellet with a minimal volume of 50 mM $NH_4HCO_3$ in 8 M urea (*see* **Note 10**).

3. Add 1 μl of endopeptidase LysC stock solution to the protein complex and incubate for 4 h at 37 °C (*see* **Note 11**).

4. Decrease urea concentration to <1.5 M by adding an adequate volume of 50 mM $NH_4HCO_3$. Add trypsin to a final concentration of 5–10 ng/μl and incubate overnight at 37 °C (*see* **Note 12**).

   Recovery of digested peptides:

5. Add 10 % formic acid to a final concentration of 1 % to stop the digestion.

6. Dry the resulting digest in a SpeedVac. Add 100 μl of water and dry again. Repeat this step one more time. Dissolve the peptide mixture in AKTA buffer A for SCX chromatography (*see* **Note 13**).

7. Separate peptides by SCX chromatography using a PolySULFOETHYL A column at a flow rate of 200 μl/min using an AKTA Basic 10.

8. Elute peptides applying a salt gradient of buffer B: 0–5 % in 2 min, 5–25 % in 20 min, 25–100 % in 10 min.

9. Collect 10–15 fractions manually based on UV absorbance at 215 nm.

10. Desalt collected SCX fractions using Vivapure C18 microspin columns following the manufacturer's instruction.

11. Analyze peptide mixtures by LC-MS/MS using nanoflow reverse phase liquid chromatography (NanoLC) coupled online to a QSTAR XL MS instrument. Elute peptides with a linear gradient of 0–35 % nano LC solvent B in 80 min at a flow of 250 nl/min. LC-MS/MS is operated in an information-dependent mode in which each full MS analysis is followed by three MS/MS acquisitions where the three most abundant peptide molecular ions are dynamically selected for collision induced dissociation (CID) to generate tandem mass spectra (*see* **Note 14**).

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### 3.4 Protein Identification and Quantification Using Protein Prospector

1. Obtain monoisotopic masses of both parent ions and corresponding fragment ions, parent ion charge states, and ion intensities from the MS/MS by using an automated version of the Mascot script from Analyst QS within Protein Prospector.

2. Use the Batch-tag program within Protein Prospector for database searching. Select trypsin as the enzyme and set the maximum number of missed tryptic cleavage sites as 2. Chemical modifications such as protein amino-terminal acety-lation, methionine oxidation, amino-terminal pyroglutamine, and deamidation of asparagine residues are selected as variable modifications. These modifications, except for protein amino-terminal acetylation, need to be chosen because of their frequent occurrence during sample preparation. For SILAC experiments, $^{13}C_6{}^{15}N_4$-arginine and $^{13}C_6{}^{15}N_2$-lysine need to be chosen as variable modifications as well. Set the mass accuracy for parent ions and fragment ions as ±200 ppm and 300 ppm, respectively. Any annotated protein databases such as SwissProt and UniProt can be used for database searching. A concatenated database composed of a normal and its reverse database can be generated in Protein Prospector for database searching. Because we purify the samples from human cell lines, *Homo sapiens* is selected as the restricted species.

3. General protein identification is based on at least two peptides with an expectation value cutoff of 0.01.

4. The SILAC ratios are calculated using the Search Compare program by calculating the relative abundance ratios of arginine/lysine-containing peptides based on ion intensities of monoisotopic peaks observed in the MS spectra at the time when the peptides are sequenced and subsequently identified during database searching. Signal to noise ratio >2 is required for peaks to be considered for quantitation. The SILAC ratios can be further validated by checking all of the raw spectra within the Protein Prospector Search Compare program. The ratio outliers are easily visualized on the ratio plots in Protein Prospector. If the peptide peaks are mixed with other peptide peaks or buried in the noise peaks, they cannot be used for quantification. The SILAC ratios are often reported as average values plus standard deviations. Only reproducible data should be reported as final results.

### 3.5 Identification of Dynamic and Stable PIPs Using PAM- SILAC and MAP-SILAC

The general workflow for PAM-SILAC and MAP-SILAC experiments is outlined in Fig. 1. For each experiment, use ten 150 mm plates of each type of cells. Perform each experiment at least twice to make sure the results are reproducible.

#### 3.5.1 PAM-SILAC Experiment

1. Lyse $293^{Rpn11\text{-}HTBH}$ cells (grown in light SILAC medium) and $293^{HTBH}$ (grown in heavy SILAC medium) using lysis buffer A.

2. Mix equal amounts of the two differentially labeled cell lysates.

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3. Carry out affinity purification using mixed lysates as described in Subheading 3.2. Use the optimal incubation time, i.e., 2 h (*see* **Note 5**).

4. Perform protein digestion, SCX separation, desalting, and LC-MS/MS analysis as described in Subheading 3.3.

5. Protein identification and quantitation as described in Subheading 3.4.

**3.5.2 Time-controlled (Tc)-PAM-SILAC Experiment—**Three separate PAM-SILAC experiments are performed by selecting three incubation times. Since the optimal incubation time is 2 h, two shorter incubation times, 20 min and 1 h, are selected. This allows the identification of dynamic proteins based on changes in their relative abundance ratios with incubation times (Fig. 2).

#### 3.5.3 MAP-SILAC Experiment

1. Lyse $293^{Rpn11\text{-}HTBH}$ cells (grown in light SILAC medium) and $293^{HTBH}$ cells (grown in heavy SILAC medium) using lysis buffer A.

2. Carry out affinity purification as described in Subheading 3.2 from equal amounts of two differentially labeled cell lysates separately. Use the optimal incubation time, i.e., 2 h (*see* **Note 5**).

3. Mix the two purified samples (*see* **Note 15**).

4. Perform protein digestion, SCX separation, desalting, and LC-MS/MS analysis as described in Subheading 3.3.

5. Carry out protein identification and quantitation as described in Subheading 3.4.

**3.5.4 Identifying Dynamic and Stable Interactors of Proteasome Complexes Based on MAP-SILAC and PAM-SILAC Ratios—**All putative proteasome-specific interacting proteins should have MAP-SILAC ratios >1.5, but not all of them have PAM-SILAC ratios >1.5. The characteristic PAM-SILAC and MAP-SILAC ratio profiles for dynamic and stable interacting proteins are illustrated in Fig. 2.

Dynamic proteasome-interacting proteins are identified when:

1. Their MAP-SILAC ratios are above a selected threshold (>1.5) [14] and are at least twofold higher than their PAM-SILAC ratios (*see* **Note 16**).

2. Their PAM-SILAC ratios increase with decreased incubation time in Tc-PAM-SILAC experiments (*see* **Note 17**).

Stable proteasome interacting proteins are identified when:

1. Their MAP-SILAC and PAM-SILAC ratios are very similar and >1.5.

2. Their PAM-SILAC ratios do not change with incubation time in Tc-PAM-SILAC experiments.

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### 3.6 Validation of Dynamic Interactions Using Quantitative Western Blotting

An alternative strategy to confirm dynamic interactions identified by MAP-SILAC and PAM-SILAC experiments is by protein co-expression, affinity purification, and quantitative immunoblotting. To illustrate the process, we choose to use the validation of a selected proteasome dynamic interactor, ADRM1, as an example. As shown in Fig. 3a, ADRM1 has the characteristic PAM- SILAC and MAP-SILAC ratio profiles for dynamic interactors. To confirm the dynamic interaction between ADRM1 and the proteasome, we examine the interaction exchange of ADRM1 during purification by expressing FLAG-tagged ADRM1 only in control $293^{HTBH}$ cells and not in $293^{Rpn11\text{-}HTBH}$ cells and by carrying out HB-based affinity purification using the Tc-PAM and MAP methods. Because proteasomes are only purified from $293^{Rpn11\text{-}HTBH}$ cells that express no FLAG-tagged proteins, any co-purification of ADRM1-FLAG using the PAM method should be the result of interactions formed in the mixed lysates during the incubation. Therefore, co-purification of ADRM1-FLAG would be expected only in PAM-purified samples but not in MAP-purified samples (Fig. 3b).

In addition, the amount of co-purified ADRM1-FLAG should increase with increased incubation time during Tc-PAM experiments. Together, this would confirm the dynamic nature of ADRM1 interaction determined by PAM-SILAC and MAP-SILAC experiments (Fig. 3a).

#### 3.6.1 Transfection of ADRM1-FLAG into Control Cell Lines

1. Transiently transfect $293^{HTBH}$ cells with pcDNA/FRT-ADRM1- FLAG [9]. Twenty-four hours after transfection, wash the cells three times in PBS and lyse the cells in lysis buffer A. Centrifuge the lysate at maximum speed of a microcentrifuge for 15 min to obtain a cleared lysate (lysate A).
2. Grow $293^{Rpn11\text{-}HTBH}$ cells similarly without transfection and lyse the cells the same way as described above to obtain a cleared lysate (lysate B).
3. Measure protein concentrations of lysates A and B, and divide equal amounts of lysates A and B into four aliquots.

#### 3.6.2 HB-tag Based Affinity Purification Using the Tc-PAM Strategy

1. Take three aliquots of lysates A and B.
2. Mix equal amounts of lysates A and B to make three aliquots of mixed lysates for PAM experiments.
3. Follow the general purification protocol described in Subheading 3.2. The incubation times for the three PAM experiments are 20 min, 1 h, and 2 h.

#### 3.6.3 HB-tag Based Affinity Purification Using MAP Strategy

1. Take one aliquot of lysates A and B.
2. Perform affinity purification as described in Subheading 3.2 from lysates A and B separately. Use the optimal incubation time (i.e., 2 h).

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3. Mix the two purified samples for subsequent immunoblotting analysis.

#### 3.6.4 Quantitative Western Blotting

1. Load the four purified samples from Tc-PAM (Subheading 3.6.2) and MAP (Subheading 3.6.3) experiments for one-dimensional SDS-PAGE. Transfer proteins to a PVDF membrane and analyze the proteins by immunoblotting.
2. Probe ADRM1-FLAG protein in the four purified samples using a mouse anti-FLAG antibody (1:2,000) followed by HRP-conjugated anti-mouse IgG (1:10,000).
3. Strip the blots by incubating the membrane in striping buffer for 30 min and re-probe with mouse anti-Rpt6 (1:1,000) followed by HRP-conjugated anti-mouse IgG (1:10,000) to detect the presence of the proteasome in the purified samples. The Rpt6 signal is used as the internal standard for normalization of proteasome loading.
4. Perform quantitative immunoblotting analysis using Cy5-conjugated anti-mouse IgG (1:10,000) as the secondary antibody. Quantify fluorescence intensities of the ADRM1-FLAG and Rpt6 bands using an Odyssey infrared scanning system (*see* **Note 18**).
5. Plot the ratios of ADRM1-FLAG to Rpt6 against incubation times to determine whether interaction exchange between endogenous ADRM1 from $293^{Rpn11\text{-}HTBH}$ cells and ADRM1-FLAG from $293^{HBTH}$ control cells during Tc-PAM experiments occurred. No interaction exchange should be observed in the sample purified from MAP experiment.

## 4 Notes

1. The HTBH-tag consists of two hexahistidine tags, a TEV cleavage site, and a signal sequence for in vivo biotinylation, which allows efficient purification of proteasome complexes in a single step by binding to streptavidin resins and specific elution by cleavage with TEV protease [13].
2. SILAC media from other brands such as Thermo Scientific should work as well.
3. Make fresh 1 mM TFA each time from a 100 mM TFA stock solution.
4. Any tandem mass spectrometer that can produce MS1 spectra with a resolution high enough to determine SILAC ratios can be used.
5. For the HTBH-tag, use 10 μl of Streptavidin beads per 150 mm plate of 293 cells for maximum specific binding efficiency with minimal background binding. Purification efficiency should be followed by western blot analysis. The optimal binding for proteasome complexes to Streptavidin beads is 2 h. The binding efficiency decreases when the incubation time decreases.
6. Effective washing steps can be achieved in micro-columns from Bio-Rad, for example, to minimize the bead loss.

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7. A Rotator in a 30 °C incubator works best for this step. Alternatively, you can perform this step at 4 °C overnight.
8. The elute can be stored at −80 °C at this point if subsequent analysis will not be carried out immediately.
9. For best results, clear siliconized tubes should be used to visualize the pellet and minimize sample loss.
10. Gradually add a small volume (e.g., 25 μl) of the buffer to dissolve the pellet and keep the volume to the minimum. It is the best not to exceed the final volume of 100 μl.
11. LysC digestion can go from 4 h to overnight.
12. Trypsin digestion can go from 8 h to overnight.
13. It is critical to minimize the salt concentration in the sample before SCX separation. It is the best not to exceed 25 mM salt before loading. If needed, desalting with C18 ZipTips or spin columns can be performed.
14. For MS instruments with fast scanning rates such as the LTQ-Orbitrap, top ten peaks can be sequenced in each LC-MS/MS acquisition cycle.
15. The SILAC ratios of background proteins should be about 1. If not, it suggests that the mixing is not equivalent and the final protein SILAC ratios need to be adjusted accordingly.
16. Comparison of MAP-SILAC and PAM-SILAC ratios alone is sufficient to identify dynamic interactors.
17. For interactors with very fast on/off rates, Tc-PAM-SILAC ratios alone cannot determine whether they are dynamic interactors. This requires MAP-SILAC experiments for unambiguous identification.
18. The Fuji imaging system works well.

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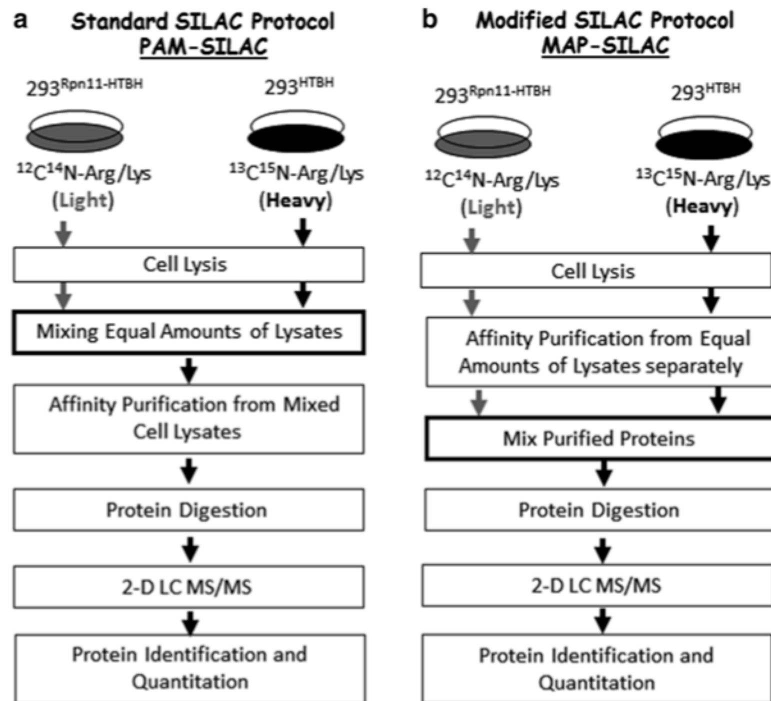


**Fig. 1.**

SILAC-based AP-MS strategies to capture and identify dynamic and stable human proteasome-interacting proteins. $293^{Rpn11\text{-}HTBH}$ cells are grown in light SILAC medium containing $^{12}C_6{}^{14}N_4$-Arg/$^{12}C_6{}^{14}N_2$-Lys (*gray* color), whereas $293^{HTBH}$ cells are grown in heavy SILAC medium containing $^{13}C_6{}^{15}N_4$-Arg/$^{13}C_6{}^{15}N_2$ -Lys (*black*). Two experimental schemes are depicted. (**a**) The standard SILAC method: PAM (purification after mixing)-SILAC; (**b**) the modified SILAC method: MAP (mixing after purification)-SILAC

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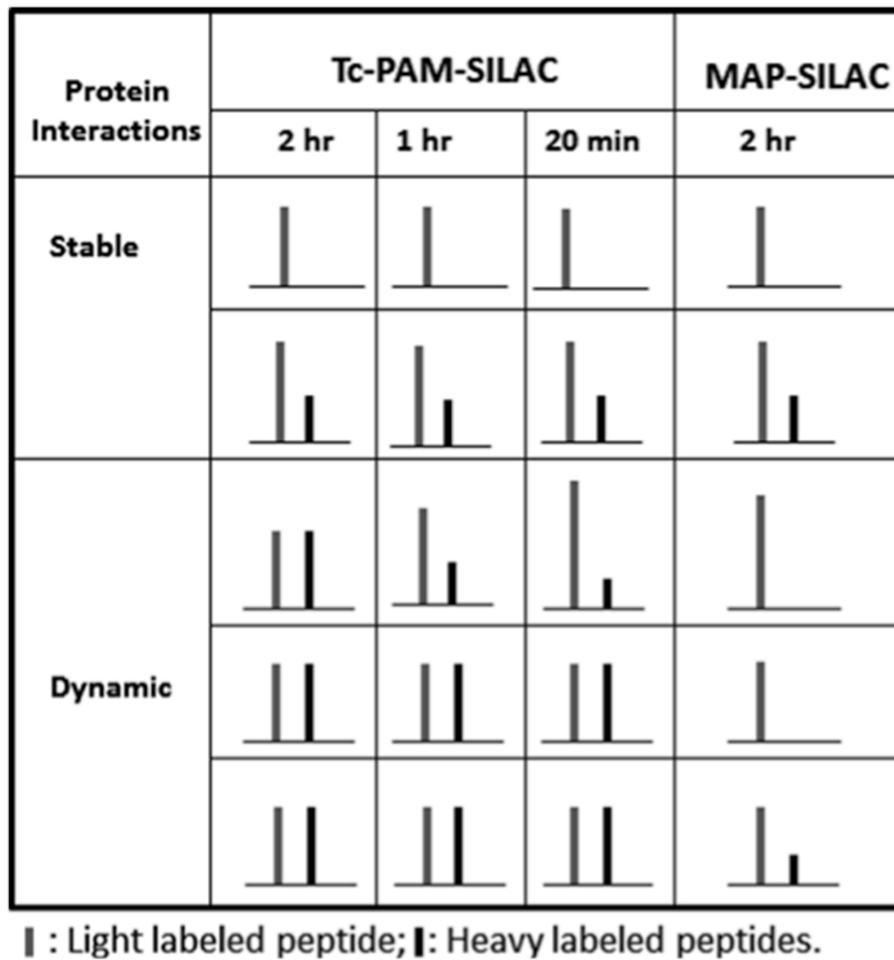


**Fig. 2.**

Characteristic PAM-SILAC and MAP-SILAC ratio profiles of dynamic and stable interactors. The relative abundance of proteins is calculated based on the ratios of mass spectral peak intensities of the observed peptide pairs colored in *gray* (light form) and *black* (heavy form). Several typical examples are shown here. *Tc*: time controlled

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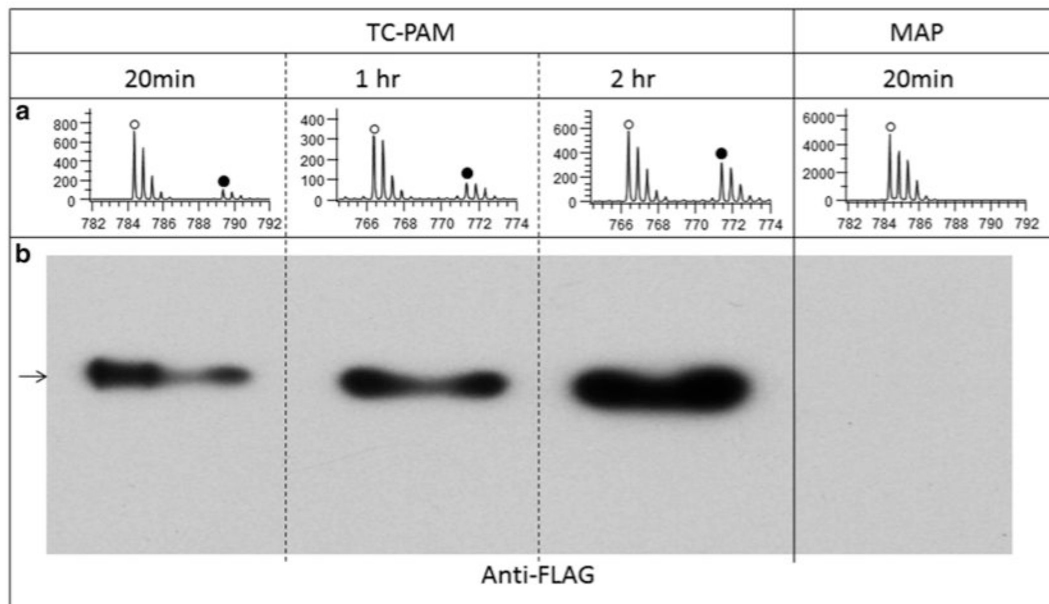


**Fig. 3.**

(**a**) TOF MS spectra of a tryptic peptide (*m/z* 766.39$^{2+}$, Acetyl-TTSGALFPSLVPGSR) matched to ADRM1/hRpn13 (a dynamic interactor). "*closed circle*" and "*filled circle* " represent the light and heavy forms of the peptide, respectively. The SILAC ratios for the peptide are shown in the corresponding spectra. As shown, its PAM-SILAC ratios increases when incubation time decreases, while its MAP-SILAC ratio is high (no heavy labeled form detected). (**b**) Validation of the dynamic interaction of ADRM1 with the proteasome using transfection, affinity purification, and quantitative western blot analysis. The band represents ADRM1-FLAG. Comparison of incorporation of ADRM1-FLAG expressed in the control cells into the purified Rpn11-HTBH containing proteasome complexes during the purification with the Tc-PAM approach at three different incubation times (20 min, 1 h, and 2 h) and with the MAP approach (2 h)